SEGREGATION, MUTATION, AND COPULATION IN SACCHAROMYCES CEREVISIAE[1]

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HAPLOPHASE CULTURES

Some individual ascospores from 4-spored asci of *Saccharomyces cerevisiae* germinate to produce haploid cells which persist in the haplophase, thus producing entire cultures containing only haploid cells. We ('43c) have reported a new method of hybridizing yeasts by mixing haplophase cells of different origin in broth. The mixtures result in copulations between different haplophase cells if each culture is paired with its complementary type. The diploid hybrid cells produced by copulation are capable of forming asci containing 4 viable spores.

Šatava ('18) and Winge and Laustsen ('37) showed that some of the single ascospore cultures from many recognized species of *Saccharomyces* contain only round, or roundish, rather small, haploid cells. They observed these cells fuse in some cultures to produce dumbbell-shaped zygotes from which elongate vigorous diploid cells arose by budding. These investigators all agree that such haploid cultures are obviously the equivalent of *Torulae*[2], and Winge and Laustsen attempted pairings of the standard species of *Torula* which were available in Copenhagen. They remarked, "In no instance did the pairing inoculations give rise to ascus formation. But this negative result was really to be expected as all Saccharomycetes appear to be asexual or homothallic." Their conclusions may be influenced by the fact that they observed copulations between haploid cells and ascospores in preparations held between slide and cover-glass so tightly that the cells spread out in two dimensions only. This gave beautiful photographs but may not have permitted sufficient freedom of contact to make preferences in copulation obvious. Our conclusions were based on genetical analyses rather than microscopic observations and were confirmed by controlled matings of haplophase cultures. They made no attempts to pair the haplophase cultures which they obtained from *Saccharomyces*. Šatava observed that ability to sporulate is restored by fusion of haplophase cells but apparently he made no direct efforts to pair haplophase cells from different cultures.

Winge and Laustsen considered the haplophase to arise by standard reduction and segregation of a diploid nucleus in the conventional manner (which seems to

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[2] From a strictly taxonomic point of view, the name "*Torulopsis*" should be used, for *Torula* had already been applied to another genus when it was first suggested by Hansen. However, since Hansen and all other investigators of industrial yeast have used "*Torula*", we are following their example in this article.

us the most acceptable view), while Šatava is rather vague about the relationship of haplophase and diplophase. Šatava found these "reduced" (haplophase) forms much different from standard diploid yeasts in their ability to produce spores and "die Zellen von normalen Form zu bilden." He attempted a classification of the degree of deviation of the haplophase from normal which agrees generally with our own observations. Winge and Laustsen were much more precise, and probably more accurate, in distinguishing between haplophase and diplophase. They observed the diplophase to originate either (1) by direct germination from a single, originally haploid, ascospore (text-fig. 2) or (2) by the delayed fusion of two haploid cells arising from the same haploid ascospore. In the former case, the nucleus in the originally mononucleate spore divided to produce two nuclei which fused in the new bud. They found cultures originating by direct diploidization to be degenerate in many ways, and this fact has been confirmed in our experience. We have observed that when a haploid ascospore germinates directly as a diploid cell to produce a small colony, it often dies before transfer can be made. On the other hand, when a single-spored ascus from a diploid colony germinates, it usually grows out directly as a diploid cell and is apparently fully vigorous and identical with the original form. In this case it seems probable that the original spore was diploid rather than haploid.

The first two types of diplophase cultures arising from haploid single ascospores are homozygous. Winge and Laustsen observed that homozygous diplophase cultures vary extremely in their ability to produce spores, and that the spores produced are generally of diminished viability, facts which we have confirmed. They stated that the haplophase never produces spores, but to this we have found some rare exceptions. However, the spores produced by the haplophase are much inferior in vigor to those arising from the diplophase. For example, one of the variants of the round-celled haploid culture *A*, described below, produced spores in round asci the same size as the vegetative cells, thus proving that the spores had arisen parthenogenetically. Four intact 3-spored asci and eight single ascospores from two 4-spored asci were isolated on nutrient medium and failed to produce viable cultures.

The simplest criterion for distinguishing haplophase from diplophase is difference in cell shape and size. This is not a perfect method although the large ellipsoidal true diploids are easily distinguished from the small spherical true haploids. However, some haploid cells are regularly elliptical but smaller than the corresponding diploid and there are many intermediate types. Inspection of many cultures has confirmed the view that many single ascospore cultures are haploid and remain haploid. Haploid cultures are much more variable than diploid cultures. Several rough variants practically always appear when a suspension of haploid cells is plated on an agar surface. Winge and Laustsen remarked on the fact that haploid colonies contain many sectors; that they are generally smaller than diploid colonies has been observed by Winge and Laustsen, and Šatava, an observation which we have confirmed. Variation in shape of cells also occurs, and

haploid colonies originating from small spherical cells often contain large numbers of balloon cells and long slender fibrous cells. Old broth cultures contain cells described as "ameboid" by Šatava. He doubtless had these characteristics in mind when he stated that the extreme reduced forms were unable to control cell shape. According to our interpretation these so-called ameboid forms in old broth cultures are copulating cells. The variability of colonial forms in haploid cultures is due to the fact that recessive mutations occurring in the haplophase are immediately revealed in the phenotype. A diploid culture may be equally mutable but the "opposite number" normal allele is dominant and the mutation does not come into expression. The mutated genes present in a normal, smooth-colonied, wild-type, diploid cell are revealed when four spores are dissected and each is grown separately. Almost invariably none resembles the parent culture.

THE "ROUGH" CHARACTER

The most useful diagnostic character differentiating various haplophase cultures is the so-called "roughness" of the colony grown on solid medium. The tendency to classify yeasts as "rough" or "smooth" is not a fruitful approach to the subject. Smoothness is the wild-type character which distinguishes the form capable of successful competition under natural conditions. It depends on the fact that the cells do not cohere to form a specific pattern, but daughter cells separate quickly from mother cells and each new unit falls smoothly into place making the colony a round hemispherical structure on the solid substrate. Roughness depends on the cells clustering in such a way that a definite pattern results when the macroscopic colony reaches its full growth. The basic pattern of aggregation is observable in microscopic examination of the cells from broth cultures, for rough types generally bud in various "rosette" forms.

Winge ('35) has described the "figure 8" arrangement of haploid cells prior to copulation (text-fig. 2). After a bud has reached full size, two new buds appear (one from the mother and one from the daughter cell) near the point of union of daughter and mother cell, producing a "4-leaf clover" effect. Most "rosettes" originate in a variation of the "figure 8" formation. Many of these cultures make what appears to be homogeneous suspensions because the "rosettes" are too small to affect the turbid appearance produced when the culture is suspended in fluid medium or grown on broth, but the extremely rough colonies cannot be easily brought into a homogeneous suspension and when rough-type yeasts are grown in liquid medium the supernatant liquid is completely clear. This is a character much desired for wine yeasts, especially for champagne yeasts. There is a basic pattern of cohesion even in extremely smooth cultures, for nearly all colonies show some distinctive topographical structure if grown on solid medium long enough to form a giant colony. Conversely, when moderately rough colonies are sown heavily enough on agar to prevent the formation of large colonies, only smooth ones appear. On an unevenly spread plate one finds an outer fringe of extremely rough, large colonies surrounding a central group of small,

smooth ones. All belong to the same genotype but the rough character cannot come into expression until the colony attains considerable size.

We have observed several hundred different types of rough-colonied yeasts, and although each one is distinctive and recognizable and can be duplicated and recognized when transplanted, we have not thus far discovered any exact duplicates. The range of variation is extremely great. In addition to the fundamental "rosette" or budding pattern, differences in shapes and sizes of the cells affect the colonial form. In all colonies the variation in cell size increases with age, generally in the direction of producing larger cells. As a rule, rough colonies contain more elongate cells than smooth colonies, and part of the basis for extreme roughness is possibly the maintenance of end-to-end connections after cell division which has been described so frequently in the genus *Bacillus*.

All four cultures from the single ascospores isolated from a 4-spored ascus originating from stable, smooth-colonied, wild-type diploid cultures of *Saccharomyces cerevisiae* are usually rough-colonied. This proves that the genes differentiating rough from smooth colonies are recessive and several loci are involved. The wild-type "opposite number" alleles of the mutant genes prevent them from coming into expression in the heterozygous wild-type diplophase. Although we have dissected many asci from the same diploid culture, practically no duplicate cultures have been found among the colonies grown from the single ascospores, which indicates that the diploid cell is heterozygous for a considerable number of mutant genes and that many loci affect the characters lumped into the so-called "rough" class.

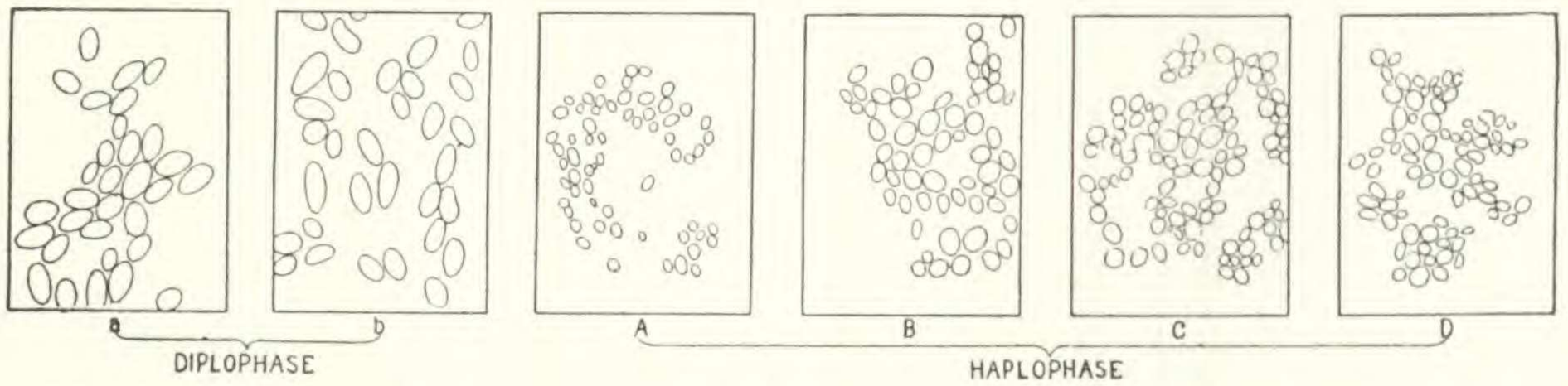
SEGREGATION AND MUTATION OF HAPLOID CULTURES

Plate 15 shows photographs of four single ascospore cultures isolated from a single ascus of the M strain. They are all in the haplophase. Usually some of the single ascospore cultures from one ascus are diploid, but we have not found any regularity in the distribution of haplophase-diplophase types indicating that the character is the result of interaction of several genes. The data presented here are merely part of an extended pedigree of the M strain, but since no new points beyond those already recorded in earlier papers were established the entire pedigree is not reported. The ascus mentioned above was derived from an inbred culture. In the inbreeding process an intact 4-spored ascus selected from the original M strain was isolated and formed a colony. This colony was plated on agar and a single colony from this plate was selected and induced to sporulate. An intact 4-spored ascus was again selected from the best sporulater, grown alone, induced to sporulate, and an ascus from this second generation was the origin of the third-generation colonies shown in pl. 15.

Plate 16, figs. a and b, show the diplophase cultures obtained from the colonies grown from two intact 4-spored asci. A study of cell shape and size of the six cultures showed that the single ascospore cultures were haploid while the two cultures obtained from intact asci were diploid. Drawings of the cells on the same

scale are shown in text-fig. 1. The diploid cells are long and ellipsoidal in contrast to the round cells from the haploid cultures.

The colonies of the diploid cultures are uniform provided they are plated on agar before spores form spontaneously. If they are left a few weeks on an agar plate and then plated again on agar, variations appear due to the germination of ascospores which have formed in the colony. The large colony size is evidence of the vigor of the diplophase.

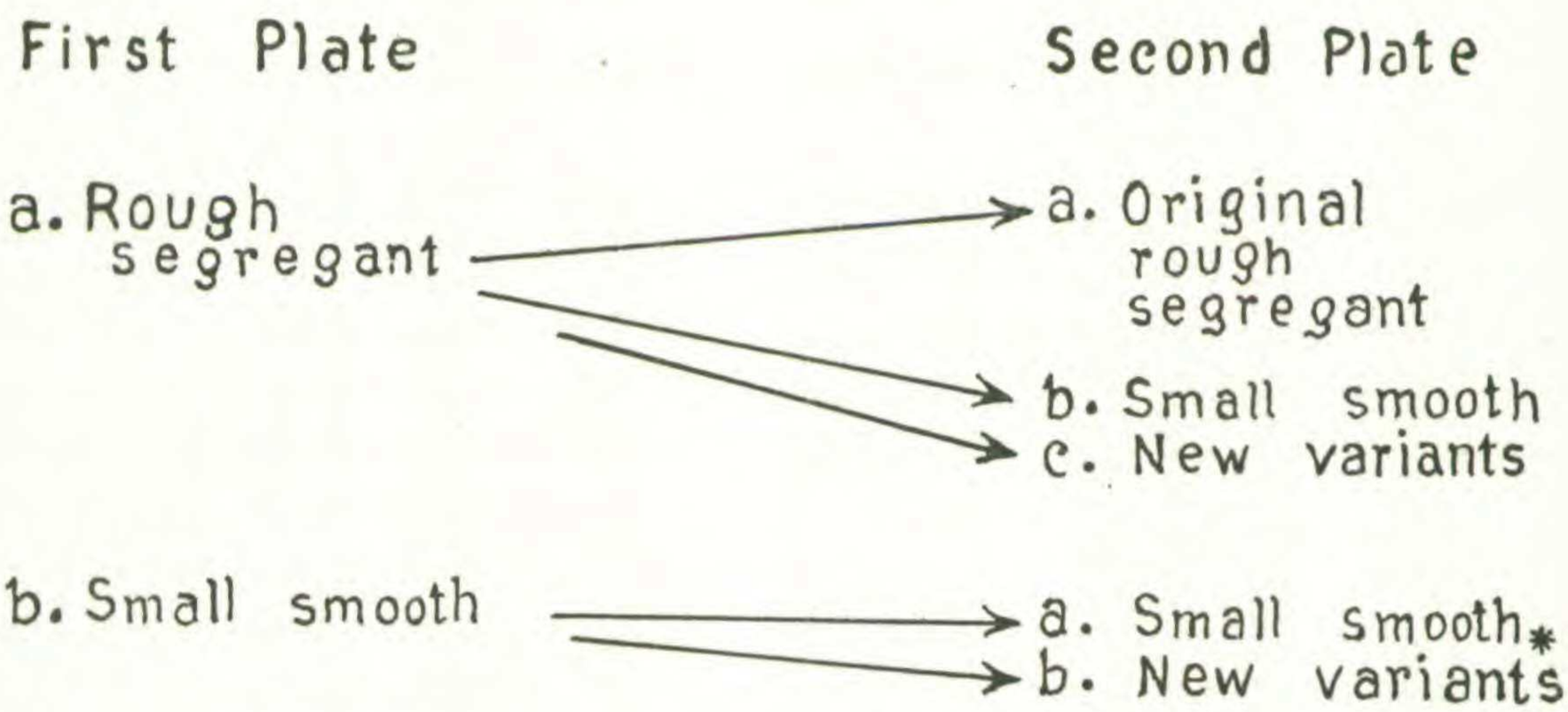


Text-fig. 1. Outline drawings (copied from photographs) of the cells from the six cultures shown in pls. 15 and 16.

The difference in colony type produced by the four haplophase cultures shown in pl. 15 are indications of the degree of heterozygosis of the diplophase from which they were derived. The original ascospores and the resultant colonies are designated *A*, *B*, *C*, and *D*. The different colony types are produced by segregation of genes at spore formation and multiplication of haploid cells, followed by mutation. These are the same cultures which were mated with each other in all combinations as previously reported (Lindegren and Lindegren, '43c). The earlier paper shows a photomicrograph of cell suspensions of the four cultures and reveals that the cells of the rough colony *(D)* produce "rosettes" characteristic of most rough colony types. Matings revealed that *A* and *D* belong to class a, while *B* and *C* belong to class *a*. Culture *A* is weakest and at the first plating produced only small colonies all of which were apparently uniform. However, in small colonies the phenotype is difficult to determine, since diagnostic characters are clearly shown only by relatively large colonies. On serial plating a large variety of colonies appeared. It does not seem profitable to report on these successive mutations since the data are simply a repetition of what has so frequently been described in bacteria. One fact was of interest, namely, that as selection and plating of colonies were continued, stable forms began to appear. These forms were all round-celled like their progenitors and therefore haploid. The weak culture *(A)* was more variable than the other three, and this is in line with observations on other strains, which indicate that a weak culture, once it is brought to a point of reasonably good growth, produces many more variations than stronger types—simply because the new mutations meet with less selection pressure from the mother colony type than in relatively more vigorous forms. As the *A* culture became better adjusted, by continued subculture, to the substrate on which it was plated, the variations became more obvious.

Cultures *B, C* and *D* show a characteristic feature of the more vigorous haplophase strains. The first plating generally shows a large rough colony accompanied by a smaller smooth form. (The cavities in the colonies reveal the places from which transfers have been made.) Transfer of the large rough colony produces the original rough type again accompanied by the small smooth form, with an occasional further departure in the shape of a new rough variant. This proves that the original rough type is the primary segregant while the small smooth form is a secondary mutation of the original segregant. This is further indicated by the fact that the small smooth form is much more stable than the original rough. Subculture of the smooth produces the smooth form almost exclusively, with only a few other variants which are often not generally comparable to the original segregant.

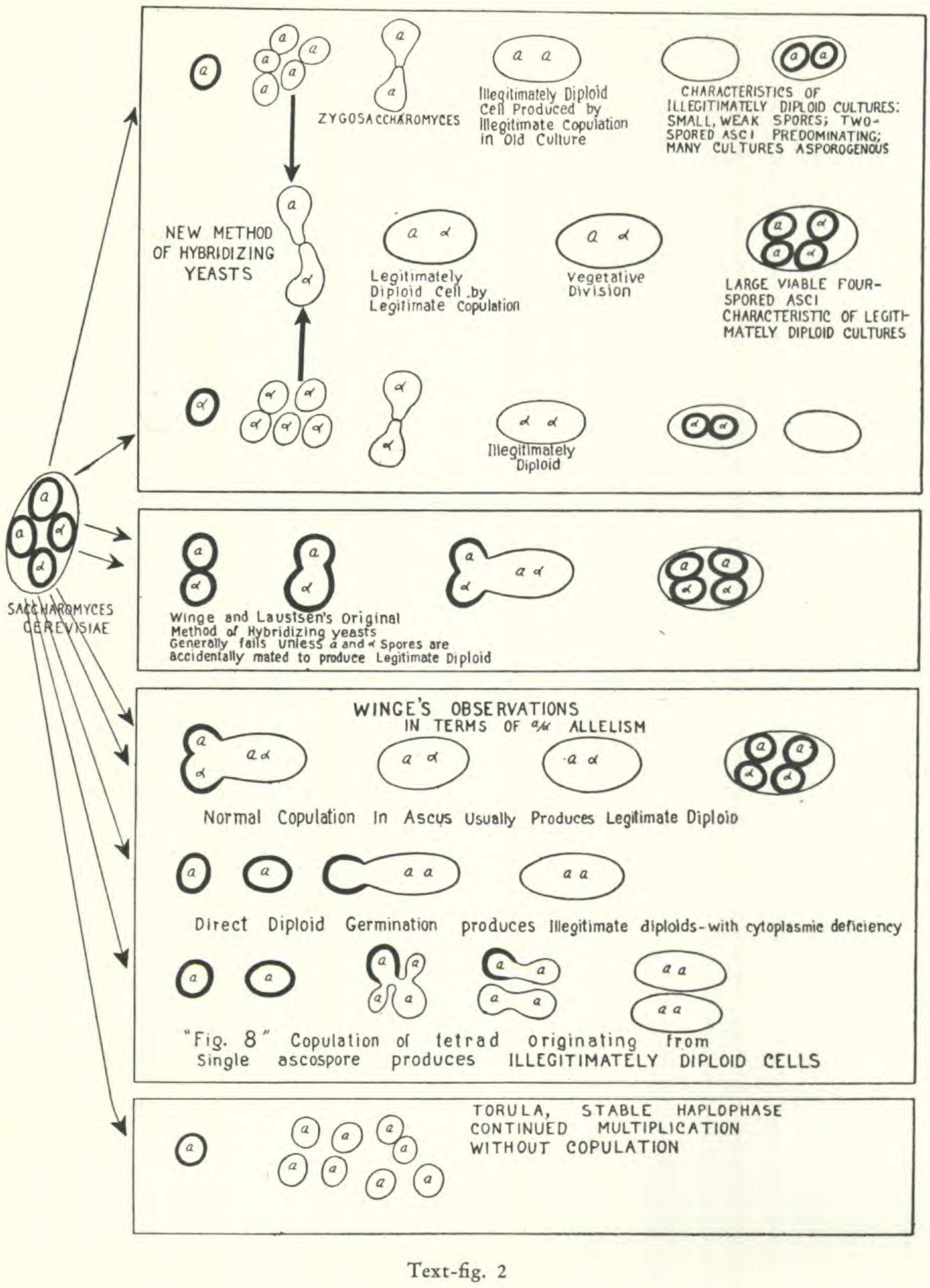
The sequence is as follows:



*These new forms are not generally comparable.

The four segregants shown in pl. 15 are characteristically different in colony shape and topography. The *D* culture is the roughest. The *C* culture produces the largest colonies and subsequent testing on a variety of sugars indicated that it was the best gas producer and the best yielder. The *A* culture, even after long subculturing, was much weaker.

Plate 17 shows the colonies of the four primary types obtained by subculture six months after the original isolation. The three photographs labelled "*A*" all show mutations of the *A* culture. Two of these are relatively stable while considerable variation is encountered in the third. The large colony size shows that some of these new variants have become fairly well adapted to the medium. The primary segregants from ascospores *B, C* and *D* are still rather clearly recognizable in spite of several serial transfers. They are generally much more stable than the *A* culture although many recognizable variants were obtained from each of them. However, so much variation occurred in the serial transfers of *A* that neither the original segregant nor the lines of descent can be traced.



Text-fig. 2

Life cycle of *Saccharomyces cerevisiae*, showing relationship with *Torula* and *Zygosaccharomyces*.

COPULATIONS BETWEEN HAPLOPHASE CULTURES OF SACCHAROMYCES CEREVISIAE

Haplophase cultures of *Saccharomyces cerevisiae* fall into two main groups which we have provisionally designated a and *a*. Each culture is made up of potential gametes which can be mated with other cells of appropriate genetic composition from another culture. As the pure haplophase cultures age, illegitimate copulations occur between two cells of the same reaction in the same culture, but these matings rarely result in the production of characteristically large diploid cells. Diploid cells produced by illegitimate copulations are easily distinguished by smaller size and diminished ability to produce 4-spored asci containing viable spores. Matings between a and *a* haplophase cultures derived from a variety of strains of *Saccharomyces cerevisiae* produce large diploid cells which sporulate to form 4-spored asci containing viable spores. The procedure is as follows: One cc. of malt-dextrose dried yeast broth is placed in a 6 x ¾-inch culture tube. A large loop of cells from a broth stock culture is introduced together with a second large loop from a different broth stock culture. The tube is kept overnight in the refrigerator at about 10° C. The next morning it is placed in the incubator at about 26° C. where it is held for 48 hours. Inspection at 24 hours incubation usually does not reveal any copulations; new copulations appear after 24 hours, sometimes rather abundantly. The reason for the small inoculation is that in old haplophase broth cultures one often finds abundant copulations. These cells do not generally survive on transfer to new broth but only the small haplophase cells propagate the culture. If large amounts of cells are mixed the old homozygous copulations might be misleading. Copulations do not generally occur in colonies on agar; broth seems much better.

Only a fraction of the single ascospore cultures produces stable haplophases. We isolated stable haplophase cultures from other strains of yeast (*L*, *FLD*, 800 and 812) and paired these with each other in all combinations. The resulting diplophase was transferred to gypsum and the culture inspected for spores with the results shown in table 1. This test indicates that the a/*a* allelism is modified by other factors. Culture 28 copulates with all other forms and produces what appeared to be viable spores with both a and *a* types. On the other hand, several of the stable haplophase cultures were unable to copulate with any of the other strains against which they were tested. This was especially true with cultures isolated from the *FLD* strain whose pedigree has been published elsewhere (Lindegren and Lindegren, "*D*" family pedigree, '43b). These facts indicate that other factors superimposed on the a/*a* alleles may produce an excessively fertile or a sterile phenotype.

COMPARISON OF THE MECHANISM ASSURING CROSS-FERTILIZATION IN YEASTS WITH THAT IN OTHER ORGANISMS

Cross-fertilization in yeasts is necessary to assure the production of viable ascospores. A principal allelic pair of genes determines the copulating type to

TABLE I

RESULTS OF PAIRING HAPLOPHASE CULTURES FROM VARIOUS SOURCES IN ALL POSSIBLE COMBINATIONS.

	M 2	L 17	812 25	M 4	L 16	L 38	800 28
M 2		—	—	+S	+S	+	+S
L 17			+N	+N	+S	+S	+S
812 25				+S	+N	+	+S
M 4					—	—	+
L 16						—	+N
L 38							+S
800 28							

which a haplophase culture belongs. The situation in yeasts appears to be different from that in other living organisms, and the present discussion points out the characteristics of the various mechanisms for insuring cross-fertilization.

1. *Self-sterility alleles.*—Most hermaphroditic flowering plants are self-sterile due to a genetic mechanism which prevents pollen shed by the flower from growing down the styles of the parent plant. A mechanism that is probably fundamentally similar prevents the sperms of an individual hermaphroditic sea squirt, *Ciona,* from fertilizing eggs produced by the ovaries of the same individual.

2. *Sexual Dimorphism.*—In higher animals and some plants sexual dimorphism insures cross-fertilization. The genetic mechanism simply operates to reduce the probability of intersexes or hermaphrodites occurring.

3. *Plus-Minus Factors.*—This mechanism in *Rhizopus* is not a sexual mechanism because no unmistakeable sex organs are involved and therefore it cannot be called a self-sterility mechanism. It is more precise to consider it a special case in which a single pair of alleles controls copulation.

4. *Neurospora.*—We formerly called the alleles in *Neurospora* plus-minus factors, but later work has shown that they resemble self-sterility alleles more

closely than the factors found in *Rhizopus*. Both plus and minus thalli contain both male and female sex organs and self-fertilization is prevented. However, since the plus and minus thalli are both haploid and the zygote is invariably heterozygous for the same pair of plus-minus alleles this mechanism differs considerably from the standard self-sterility mechanism found in flowering plants in which a large series of multiple alleles exists and a great variety of heterozygotes abound.

5. *Hymenomycetes.*—The hymenomycete mechanism resembles the plus-minus *Rhizopus* mechanism rather closely since no obvious sex organs exist in these forms. It differs in the facts that in many hymenomycetes two loci are involved and that a multiple series of alleles at these loci further complicates the picture.

6. *Mating types.*—The mechanism which assures cross-fertilization in the single-celled diploid *Paramecium* resembles the plus-minus mechanism found in fungi since no sex organs are present, but the heredity seems to be more complex. The fact that the copulating cells are diploid is a still further difference from the most closely comparable fungal mechanism.

7. *Yeast.*—The mechanism in yeast is clearly different from the preceding. Our experiments indicate that monohybrid allelism modified by other factors controls copulation and we have given the symbol a/α to the principal allelic pair. However, the zygote is so much more vigorous than either parent that the diploid state produced by copulation has the character of hybrid vigor. In addition to insuring cross-copulation, heterozygosis for these factors results in a great increase in vigor, especially with regard to cell size and spore viability. The diminished vigor of the haplophase assures eventual supremacy of the hybrid in competition. Yeasts are quite different from most fungi since they propagate both as haploid and diploid cells and, in nature, balance, selection, and competition occur between these states. When the spores germinate in the spring the first growth may be haploid with one haplophase type competing with another until either legitimate or illegitimate copulation occurs, producing an even more complex state of competition. In the late fall sporulation occurs. Only the legitimate diploids produce viable spores and the cycle begins in the following year after these spores have germinated.

SACCHAROMYCES, TORULA, AND ZYGOSACCHAROMYCES

The diagram (text-fig. 2), showing the life cycle of *Saccharomyces cerevisiae* and including *Torula* and *Zygosaccharomyces* as phases of the cycle, has developed from the study of single-ascospore cultures. Stable haplophase cultures derived from S. *cerevisiae* fall into three classes: (1) those which do not copulate with any other strain, (2) those which copulate with all other copulating strains, and (3) those which copulate with their allelic partners only. The first type is a round-celled *Torula*, and Šatava ('34) pointed out that this genus is invalid because all *Torulae* have probably arisen from *Saccharomyces*. Illegitimate copulations occur in most old broth cultures of classes 2 and 3. Although the capacity of these illegitimate

homozygotes to produce spores is quite variable some of the cultures sporulate rather abundantly. They would be classified as *Zygosaccharomyces*. The spores in *Zygosaccharomyces* are probably inviable, but there are few, if any, records on the viability of spores in this genus. *Zygosaccharomyces* colonies are nearly always the rough type (Lochhead and Farrell, '31), another indication supporting the view that this genus contains single ascospore cultures of *Saccharomyces*. Conditions of copulation and sporulation seem in general to parallel each other, and illegitimate homozygotes which sporulated immediately on copulation would give the classical *Zygosaccharomyces* picture.

Dvornik ('38) recently studied spore formation in 50 strains of *Saccharomyces apiculatus*. They all formed one-spored asci. The vegetative cells bud characteristically with what appear to be abortive copulation tubes and resemble very closely some of the haplophase single ascospore cultures which we regularly obtain from *Saccharomyces cerevisiae*. The genus *Nadsonia* produces a peculiar type of polar budding similar to the "rosettes" or "figure 8" conformations found in single ascospore cultures. This genus may also be a peculiar type of single ascospore culture. These considerations indicate the inadvisability of giving generic or specific rank to peculiar types of yeasts incapable of producing asci containing four viable spores.

SUMMARY

Haplophase ascospore cultures derived from 4-spored asci of *Saccharomyces cerevisiae*, when paired with their complementary types, copulate to reproduce the original form.

Haplophase cultures originating from single ascospores of *Saccharomyces* resemble *Torulae* or *Zygosaccharomyces*. They are generally "rougher" and weaker than the diploid parent. The basis for the different rough characters is the inheritance of different types of budding and cell association. Variation of the haplophase is much greater than of the diplophase and the original segregant can be differentiated from the secondary mutants by serial plating.

The factors differentiating the copulating types seem to differ fundamentally from factors insuring cross-fertilization in other forms and were designated the a/α alleles.

Torula ("Torulopsis") and *Zygosaccharomyces* are probably invalid genera and merely represent phases in the life cycle of *Saccharomyces*.

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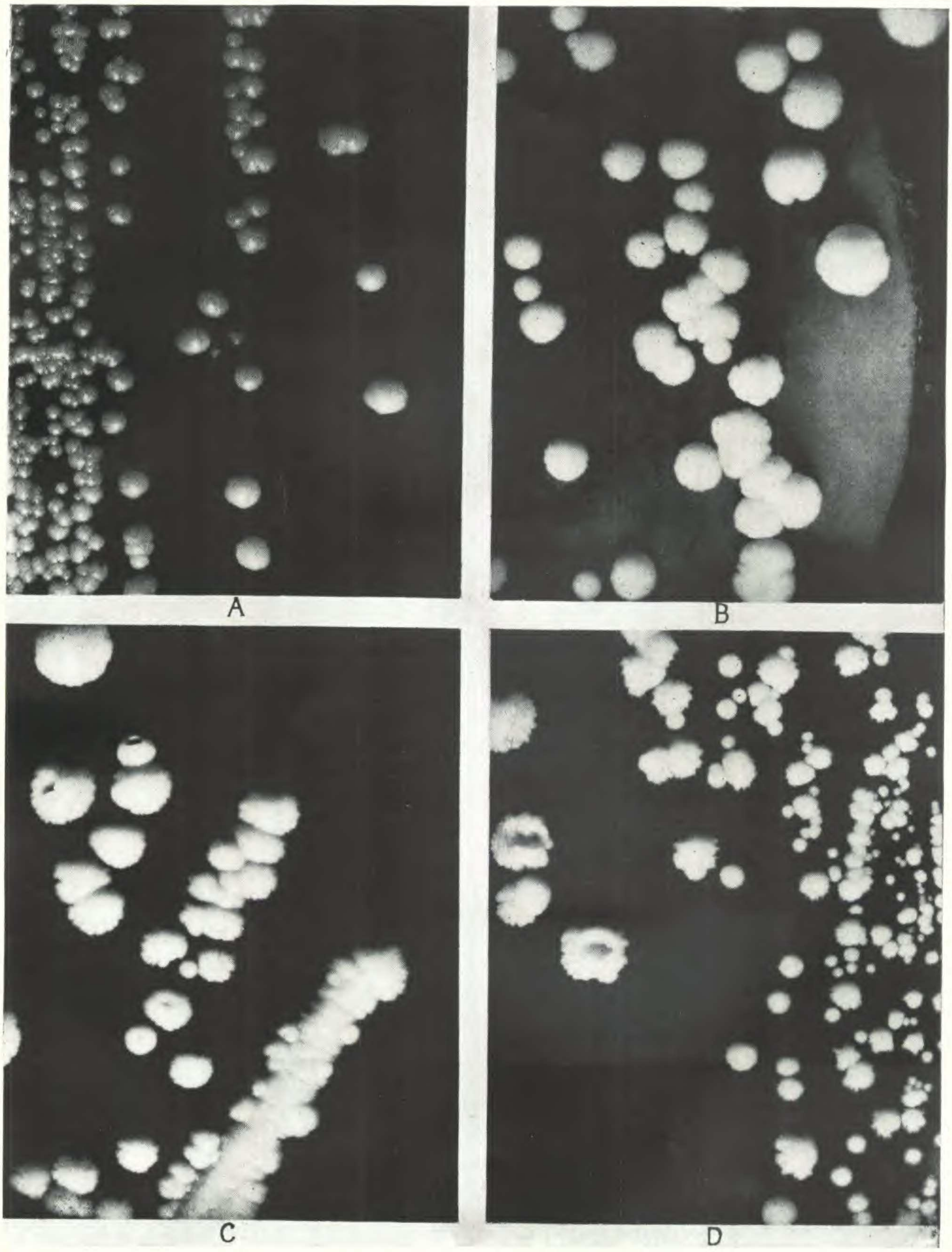
Explanation of Plate

PLATE 15

Saccharomyces cerevisiae

Colonies grown from the four ascospores dissected from a single ascus of the M strain.



LINDEGREN & LINDEGREN—SACCHAROMYCES CEREVISIAE

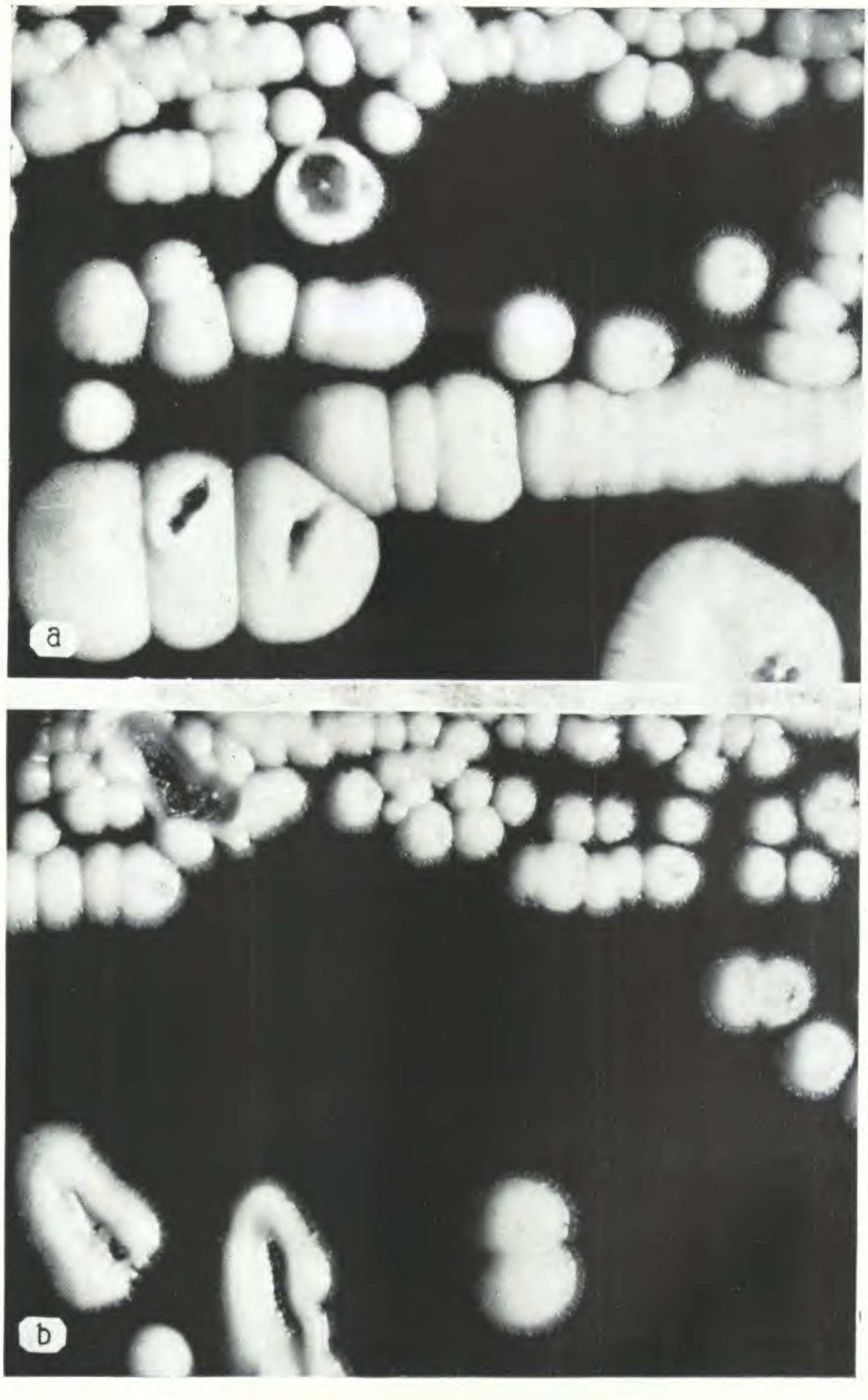
Explanation of Plate

PLATE 16

Saccharomyces cerevisiae

Fig. a. Diploid colonies produced by the self-fertilization of an intact 4-spored ascus from the same culture tube as the ascus from which spores *A*, *B*, *C*, and *D* of pl. 15 were obtained. When an intact 4-spored ascus is isolated and permitted to germinate, copulations usually occur before growth has progressed very far and the diploid cells soon outgrow the haploid ones. Diploid colonies are uniform and smooth in contrast to the haplophase colonies, which are generally rough and variable. These diploid cultures produce viable 4-spored asci while the cultures shown in pl. 15 do not.

Fig. b. A second culture obtained by self-fertilization of a 4-spored ascus in the same manner. Some cultures produced in this way are rough and cannot produce 4-spored asci. This is presumably because only a single spore in the ascus was viable.



LINDEGREN & LINDEGREN—SACCHAROMYCES CEREVISIAE

Explanation of Plate

PLATE 17

Saccharomyces cerevisiae

Haplophase subcultures of the cultures shown in pl. 15 are reisolated six months after the original cultures had been grown from ascospores.